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Phosphatidylhydroxyalkanols as a Versatile Intermediates in the Synthesis of Headgroup Modified Diacetylenic Phospholipids

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Alok Singh, Michael Markowitz and Li-I Tsao

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Alok Singh, Michael Markowitz and Li-I Tsao

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Alok Singh*, Michael Markowitz and Li-I Tsao

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Phospholipase D extract (from savoy cabbage) mediates exchange of omega hydroxyalkanols (n= 2-4) with the choline moiety of phosphatidylcholines to produce corresponding phosphatidylhydroxyalkanols in >70% yield. The phosphatidyl hydroxyalkanol and its chloro analogue were further reacted with molecules containing carboxylic and amine functionality respectively, providing an easy and efficient method to produce headgroup modified phospholipids.

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In our laboratory, we are developing technological applications[1-6] of polymerizable phospholipids. In particular, the role of diacetylene moiety in the stabilization, as well as in the modulation, of macroscopic properties of membrane self assemblies is being studied. This report is focussed on the synthesis of a variety of head group modified diacetylenic phospholipids in which charge neutral phosphocholine is replaced with reactive functionalities to provide

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*To whom correspondence should be addressed

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sites for membrane-surface modification.

We have considered a hybrid approach utilizing both enzymatic and organic routes which involve simple and high yield synthetic steps, and milder reaction conditions that are compatible with thermal- and/or photo-sensitive polymerizable moieties. Phospholipase D mediated transphosphatidylation of cholines with alkanols[7-13] constitutes a straightforward route (Scheme i). The scheme as such is not synthetically attractive due to low yields of reaction products and dependence of transformations on reaction conditions and the nature of reactants.[10-13] For example, enzyme derived from cabbage exchanges short chain but not long chain alkanols[7,8,12] with choline moiety of natural and synthetic phospholipids while the enzyme isolated from rice germ[10,11] facilitated alkanols exchanges independent of their chain length. Phospholipase D extracted from streptomyces catalyzed the transfer of higher alcohols[12] with natural phosphocholines, but remain ineffective on synthetic phosphocholines, mixed chain cholines and bulky choline groups[9]. Similarly, pure enzyme from cabbage or peanut did not produce reproducible yields of diacetylenic phosphatidyl ethanolamine or -bromoethanol.

The present scheme overcomes these problems by involving enzyme mediated synthesis of phosphatidyl hydroxyalkanols in the first step and chemical reactions with hydroxy derivatives to produce headgroup modified phospholipids in the subsequent steps. Thus, the ability of crude enzyme extract from savoy cabbage to provide high, reproducible yields of polymerizable diacetylene phosphatidic acid[14] is extended to alkanediols. The crude phospholipase D extract transphosphatidylation produced reproducible and nearly quantitative yields of hydroxyalkanols (5a-c). The yields were, however, lower in the case of diethylene glycol (4) indicating the limitations of reaction.

These hydroxy derivatives were used as reactive intermediates to produce esters and chloro analogue of the phosphoalkanol, 7. The chlorinating reagent N,N,2- trimethyl-1-chloropropenylamine[15,16] converted hydroxy lipids into

halogenated analogues in high yields (Scheme iii). Hydroxy analogue was reacted with maleic anhydride to produce ester linked carboxyl terminated lipid, 8. The halolipids reacted with alkylamines to give phospholipids 9 and 10 in good yield.

The results presented here, demonstrate the effectiveness and convenience of the scheme in preparing headgroup modified lipids. The scheme may find broader applicability in modifying phospholipids because of a) long shelf-life of phospholipase D from cabbage extract (one month at -20°C), b) almost quantitative enzymatic transformations and c) mild reaction conditions.

EXPERIMENTAL

Materials and Methods: Phospholipase D was extracted from white leaves of cabbage following the procedure reported by Eibl and Kovatchev.[7] The protein content in the extract was measured (1.7 mg/ml) following a reported method.[17] The enzyme extract was stored in a freezer and used as such. The extract remained active for one month. Diacetylenic phospholipid 1,2 bis (tricosa-10,12-diynoyl)-sn-glycero-3-phosphocholine ($DC_{8,9}PC$) was synthesized in our laboratory following the published procedures.[18-20] Ether was dried over calcium chloride. Acetate buffer (pH 5.6) containing 0.2 M sodium acetate and 0.08 M calcium chloride was used in the enzyme catalyzed reactions. Ethylene glycol, propane diol, butanediol, ethylene diaminetetraacetic acid (EDTA) were obtained from Aldrich Chemical Company. For efficient transformations, the enzyme extract:buffer ratio was kept at 1.75:1, the volume of ether was kept three times that of the acetate buffer, and the phospholipid concentration was maintained at 0.66 % of the total volume in the reaction flask.

The course of the phospholipase reaction was monitored by TLC on silica gel (Merck) employing two solvent systems; chloroform:methanol:water (65:25:4) (A) and chloroform:methanol:ammonia (25 % in water) (65:30:3) (B). Spray reagents phosphomolybdic acid was made in the lab according to established

procedure and Dragendorff's reagent were purchased from Sigma chemical company. N,N,2-trimethyl-1-Chloro-propenylamine was synthesized following the literature procedure.[15,16] Infrared spectra were obtained using a Perkin-Elmer 1800 FT-IR. NMR spectra were obtained in $CDCl_3$ using a Varian EM 390 or Brucker MSL 360 nuclear magnetic resonance spectrometer. Mass spectral analysis was carried out by fast atom bombardment (FAB) mass spectrometry using a Finnigan triple quadrupole mass spectrometer to insure both the molecular identity and the absence of calcium ions in the sample. The latter interfered in chlorination of hydroxyalkanol.

General Procedure for the Synthesis of Phosphatidylhydroxyalkanol: 500 mg (0.55 mmols) of 1,2 bis (tricosa 10,12 diynoyl)-sn-3-glycerophosphocholine, $DC_{8,9}PC$ 1, was dissolved in 30 mL of ether with gentle warming. A 100 fold excess of the appropriate diol was added. At 40°C a translucent solution was obtained. Then, a mixture of the enzyme extract (30 mL) and 0.2 M aq. NaOAc-AcOH buffer (80 mM $CaCl_2$, pH 5.6) (17 mL) was added. A pinkish color developed. An additional 20 mL ether was added. The reaction mixture was stirred vigorously at 40°C. The course of the reaction was monitored by thin layer chromatography on silica plates using solvent systems A and B. Phosphatidylhydroxyalkanols were revealed at Rf 0.53 as compared with Rf 0.33 for PC in solvent A and in solvent B R_fs were 0.69 and 0.17 respectively. The reaction mixture was stirred for 10 hours. During this time, the reaction mixture was protected from light. The ether was evaporated under reduced pressure and 100 mL of a saturated aq. EDTA solution (pH 8.5) was added to the remaining aqueous phase. The lipid was extracted thrice with a 2/1 $CHCl_3/CH_3OH$ (v/v) mixture. The organic fractions were combined and the solvent was evaporated under reduced pressure at 45°C. The residue was redissolved in a minimum amount of $CHCl_3$ and the purity of the lipid was analyzed by thin layer chromatography (solvent A). The developed plates were analyzed using

Dragendorf's reagent to monitor the disappearance of the PC. The appearance of the product lipid was monitored with phosphomolybdate reagent and iodine vapor. Any unreacted $DC_{8,9}PC$ was removed by chromatography on a silica column using the following gradient: 2 col. vols $CHCl_3$; 2 col. vols 19/1 $CHCl_3$/MeOH; 3 col. vols. 9/1 $CHCl_3$/MeOH. To insure the absence of any ion the lipids were dissolved in $CHCl_3$ and treated with an ion exchange resin (Biorad AG 50W-X8). The lipid was then dissolved in a minimum amount of warm $CHCl_3$ and precipitated at 0°C with acetone.

Synthesis of $DC_{8,9}$ Phosphatidic Acid (2): Lipid 1, (465 mg,0.51 mmol), was placed in a 100 mL single necked round bottom flask and dissolved in 3 ml of isopropanol with gentle warming. 40 mL of acetate buffer and phospholipase D extract (20 ml of enzyme extract mixed with 20 mL of acetate buffer, pH 5.6), was added to this solution. A cloudy suspension resulted which cleared upon addition of 20 mL anhydrous ether. The reaction mixture was stirred vigorously at 37°C for 10 hours protected from direct fluorescent light. Thin layer chromatographic analysis indicated the absence of phosphatidylcholine. Then, the ether was removed under reduced pressure and lipid was extracted. Combined chloroform extracts were washed once with 25 mL EDTA (50 mM, pH 8.5), and twice with water. Solvent removal yielded 450 mg phosphate positive crude lipid. Acetone precipitation provided pure lipid as a transparent waxy material (404 mg, 96% yield). ^1H NMR ($CDCl_3$) δ 0.88 (t, 6H,-$\underline{CH_3}$), 1.22-1.44 (s, 44H) merged with 1.44-1.63 (m, 12H total 56H, -($\underline{CH_2}$)-), 2.21-2.35 (m, 12H, -C≡C-$\underline{CH_2}$- and O-C(O)$\underline{CH_2}$-), 3.99 (broad s, 4H, -$\underline{CH_2}$-O), and 5.25 (m, 1H,-$\underline{CH}$-O-).

Synthesis of $DC_{8,9}$ Phosphatidylethanolamine (3): 500 mg (0.55 mmol) 1 was reacted with ethanolamine hydrochloride, following the reported procedure[3], to provide 48 mg (10% yield) of pure 3 as waxy material. The reaction provided variable yields of 3 on repeat experiments. The product was identified on TLC

plates by amine positive ninhydrin spray. Mass spectral analysis revealed parent ion peak at 870.6 (M-1).

Phosphatidyldiethyleneglycol (4): Lipid, 1, 500 mg (0.55 mmol) was reacted with 5.8 g (55 mmol) of diethylene glycol in 50 mL of dry ether and 47 mL enzyme-buffer solution (made by mixing 30 mL of phospholipase D extract and 17 mL of acetate buffer)according to the general procedure. The contents were stirred at 37°C in the dark. After reaction work-up and chromatography, 4 was collected in 25% yield. ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H,-CH_3), 1.26 (sharp singlet merged with multiplet, 44H) and 1.46-1.53 (m, 12H) for -(CH_2)-, 2.22-2.29 (m, 12H, -C≡C-CH_2- and O-C(O)CH_2-), 3.65-3.81 (m, 8H, -CH_2-O-, -CH_2-OH), 4.03 (m center, 4H, -CH_2-O-P-O-CH_2-), and 5.25 (m, 1H,-CH-O-).

Synthesis of $DC_{8,9}$ Phosphatidylhydroxyethanol (5a): Following the general procedure lipid, 1, 400 mg (0.44 mmol) was reacted with 2.83 g (45 mmol) ethylene glycol and 50 mL of dry ether in the presence of 47 mL enzyme-buffer solution (made by mixing 30 ml of phospholipase D extract and 17 mL of acetate buffer). The contents were stirred at 37°C. After the usual work-up, 390 mg crude lipid was obtained which after purification afforded 300 mg (yield 77 %) of pure product. TLC analysis using solvent A revealed the homogeneity of the compound (R_f= 0.53). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-CH_3), 1.22-1.44 (s, 44H) merged with 1.44-1.63 (m, 12H total 56H, -(CH_2)-), 2.21-2.35 (m, 12H, -C≡C-CH_2- and O-C(O)CH_2-), 3.75 (s, 2H, -CH_2-OH), 3.99 (s, 4H, -CH_2-O) 4.12-4.26 (m, 1H, -H-CH-O-), 4.34 (dd, J=4.2 and 11.9 Hz, 1H, -HCH-O-), and 5.25 (p, 1H,-CH-O-). Negative ion mass spectra produced parent ion peak at 871.3 (M-1)

Synthesis of $DC_{8,9}$ Phosphatidylhydroxypropanol (5b): Following the general procedure 500 mg 1 in 50 mL of dry ether was reacted with 3.95 mL of 1,3

propanediol in the presence of phospholipase D extract in acetate buffer (17 mL acetate buffer added to 30 mL enzyme extract). Upon workup and acetone precipitation a quantitative yield of 5b was obtained. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-CH_3), 1.2-1.44 (m with sharp singlet, 46H) and 1.44-1.63 (m, 12H) total 58H, -(CH_2)-), 2.21-2.35 (m, 12H, -C≡C-CH_2- and O-C(O)CH_2-), 3.70 (s, 2H, -CH_2-OH), 4.04-4.3 (broad m, 6H, -CH_2-O), and 5.25 (m, 1H,-CH-O-).

Synthesis of $DC_{8,9}$ Phosphatidylhydroxybutanol (5c): Following the general procedure, 500 mg of 1 in 50 mL dry ether was reacted with 4.84 mL of 1,4-butanediol in the presence of 30 mL phospholipase D extract diluted with 17 mL acetate buffer. Workup followed by acetone precipitation provided quantitative yield of 5c. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-CH_3), 1.2-1.44 (m with a sharp singlet, 48H) and 1.44-1.63 (m, 12H) total 60H, -(CH_2)-), 2.24 and 2.33 (t center, J= 6.9 Hz, 8H and J=7.1 Hz, 4H for -C≡C-CH_2- and O-C(O)CH_2- resp.), 3.71 (t, 2H, -CH_2-OH), 4.04-4.21 (m, 4H, -CH_2-O), 4.36 (d, 2H, J=11.9 Hz, 1H, -HCH-O-), and 5.25 (m, 1H,-CH-O-).

Synthesis of $DC_{8,9}$ Phosphatidylglycerol (6): Following the general procedure, 538 mg (0.59 mmol) of 1 was reacted with 5.31 gm (59 mmol) glycerol dissolved in 48 mL ether in the presence of enzyme extract and buffer. After work up and chromatography on a silica gel column, 290 mg (54 % yield) lipid 6 was obtained as light yellow wax. NMR chemical shifts were the same as that of 2 except for the 2 and 8 protons under δ 5.25 and 4.04-4.3 ppm respectively.

Synthesis of $DC_{8,9}$ Phosphatidyl 2-chloroalkanols (7): Synthesis of chloroalkanols was carried out by reacting 5a and c with 1-chloro-N,N,2-trimethyl propenylamine[17,18] (11) in chloroform-*d*. The course of the reaction was followed by NMR. In the case of 5a, disappearance of chemical shift at δ 3.75 (-CH_2-OH)

and appearance at δ 3.66 (-$\underline{CH_2}$-Cl) was observed. In the case of 6c, the ratio of chemical shifts due to -$\underline{CH_2}$-OH (δ 3.71) and -$\underline{CH_2}$-Cl (δ 3.59) was measured to monitor the course of the reaction. In both the cases, reaction was found to be complete in 30 minutes. TLC analysis using solvent A revealed the complete absence of 5a or 5c (R_f of 7 is 0.61).

Preparation of $DC_{8,9}$ Phosphatidyl 2-Chloroethanol (7a): Phospholipid 5a (400 mg, 0.46 mmol) was reacted with 11 (400 mg, 3 mmol) in 4 mL of freshly distilled chloroform (distilled over P_2O_5). The reaction mixture was carried out at room temperature under nitrogen. The course of reaction was monitored by TLC using solvent A. After completion of the reaction, the excess chloroform was removed and the residue was chromatographed on a column of silica gel. Elution with chloroform-methanol (9:1) provided 374 mg 7a as white wax in 91% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-$\underline{CH_3}$), 1.22-1.44 (s, 44H) merged with 1.44-1.63 (m, 12H, total 56H, -($\underline{CH_2}$)-), 2.21-2.35 (m, 12H, -C≡C-$\underline{CH_2}$- and O-C(O)$\underline{CH_2}$-), 3.66 (s, 2H, -$\underline{CH_2}$Cl), 3.99 (s, 4H,-$\underline{CH_2}$-O) 4.12-4.44 (m, 2H,-$\underline{H}$-C$\underline{H}$-O-), and 5.25 (m, 1H,-$\underline{CH}$-O-).

Synthesis of $DC_{8,9}$ Phosphatidyl 2-(Hydroxyethyl)-maleic acid (8): Phospholipid 5a, 49.0 mg (0.056 mmol) was reacted with 40 mg (0.4 mmol) maleic anhydride in 1 mL pyridine. After stirring at room temperature for overnight most of the starting material was found consumed with an emerging new spot at lower Rf on TLC plate (solvent A). The product was dissolved in 2:1 chloroform/methanol and the pyridine was removed by washing with 10% aq. copper sulfate. After removing all the pyridine, the lipid solution was washed with 2% hydrochloric acid and the solvent was removed. The residue was chromatographed on a column of silica gel to afford 23 mg of 8 (42 % yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-$\underline{CH_3}$), 1.22-1.44 (s, 44H) merged with 1.44-1.63 (m, 12H total 56H, -($\underline{CH_2}$)-), 2.21-2.35 (m, 14H, -C≡C-$\underline{CH_2}$- and O-C(O)$\underline{CH_2}$-), 4.02-

4.45 (m, 6H, -$\underline{CH_2}$-O-), 5.25 (m, 1H,-$\underline{CH}$-O-), 6.28 (d, $\underline{J}$= 12.6 Hz, 1H, -$\underline{CH}$=C-), and 6.4 (d, $\underline{J}$= 12.6 Hz, 1H, -C=$\underline{CH}$-).

Synthesis of $DC_{8,9}$ Phosphatidyl *N*-Methylaminoethanol (9): In a teflon capped reaction tube, 30 mg (0.034 mmol) of 7a dissolved in methylene chloride was reacted with excess of dry methylamine dissolved in the same solvent. The contents were stirred at room temperature in tightly closed reaction tube. TLC analysis using solvent system A revealed the disappearance of starting material and emergence of new spot at R_f 0.40 within three hour. After 4 hours of standing at room temperature TLC analysis revealed the appearance of a slow moving spot. The reaction was stopped by removing solvent and methylamine by rotary evaporation. The mixture was separated on a column of silica gel (elution with chloroform-methanol, 9:1). During work up and chromatography steps some hydrolysis was observed. Due to this reason variable yields were obtained while the minimum yield being 30%. The slow moving spot was identified as the lyso analogue by NMR. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-$\underline{CH_3}$), 1.22-1.44 (s, 44H) merged with 1.44-1.63 (m, 12H total 56H, -($\underline{CH_2}$)-), 2.21-2.35 (m, 12H, -C$\equiv$C-$\underline{CH_2}$- and O-C(O)$\underline{CH_2}$-), 3.01 (s, 3H, $\underline{CH_3}$-N), 3.43 (s, 2H, -$\underline{CH_2}$-N), 3.99 (s, 4H, -$\underline{CH_2}$-O), 4.12-4.40 (m, 2H, -$\underline{H}$-C$\underline{H}$-O-), and 5.25 (p, 1H,-$\underline{CH}$-O-).

Synthesis of $DC_{8,9}$ Phosphatidyl *N,N*-Dimethylaminoethanol (10): In a teflon capped reaction tube, 30 mg (0.034 mmol) of 7a dissolved in methylene chloride was reacted with excess of dry dimethylamine dissolved in the same solvent. The reaction mixture was tightly closed in a reaction tube and stirred at room temperature. Within one hour, TLC analysis (solvent system A) revealed the disappearance of 7a and emergence of a new spot at R_f 0.57 due to 10. After 4 hours of standing at room temperature TLC revealed the appearance of a spot moving at R_f 0.25 (lyso analogue). The reaction products were separated on a

column of silica gel (elution with chloroform-methanol, 9:1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (t, 6H,-$\underline{CH_3}$), 1.22-1.44 (s, 44H) merged with 1.44-1.63 (m, 12H total 56H, -($\underline{CH_2}$)-), 2.21-2.35 (m, 12H, -C≡C-$\underline{CH_2}$- and O-C(O)$\underline{CH_2}$-), 2.94 (s, 3H, -$\underline{CH_3}$-N), 3.01 (s, 3H, $\underline{CH_3}$-N), 3.43 (s, 2H, -$\underline{CH_2}$-N), 3.99 (s, 4H, -$\underline{CH_2}$-O), 4.12-4.40 (m, 2H, -$\underline{H}$-C$\underline{H}$-O-), and 5.25 (p, 1H,-$\underline{CH}$-O-). Negative ion mass spectrum revealed parent ion peak at 898.5 (M-1).

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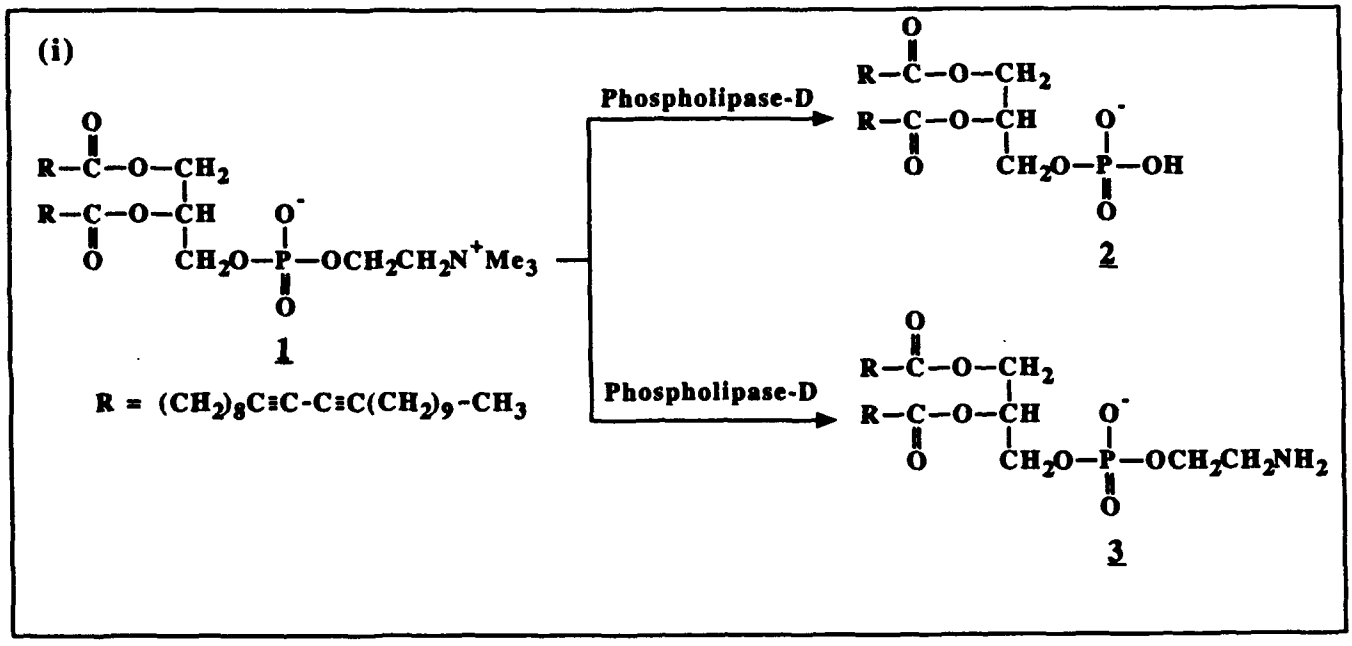
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Phospholipase-D
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3
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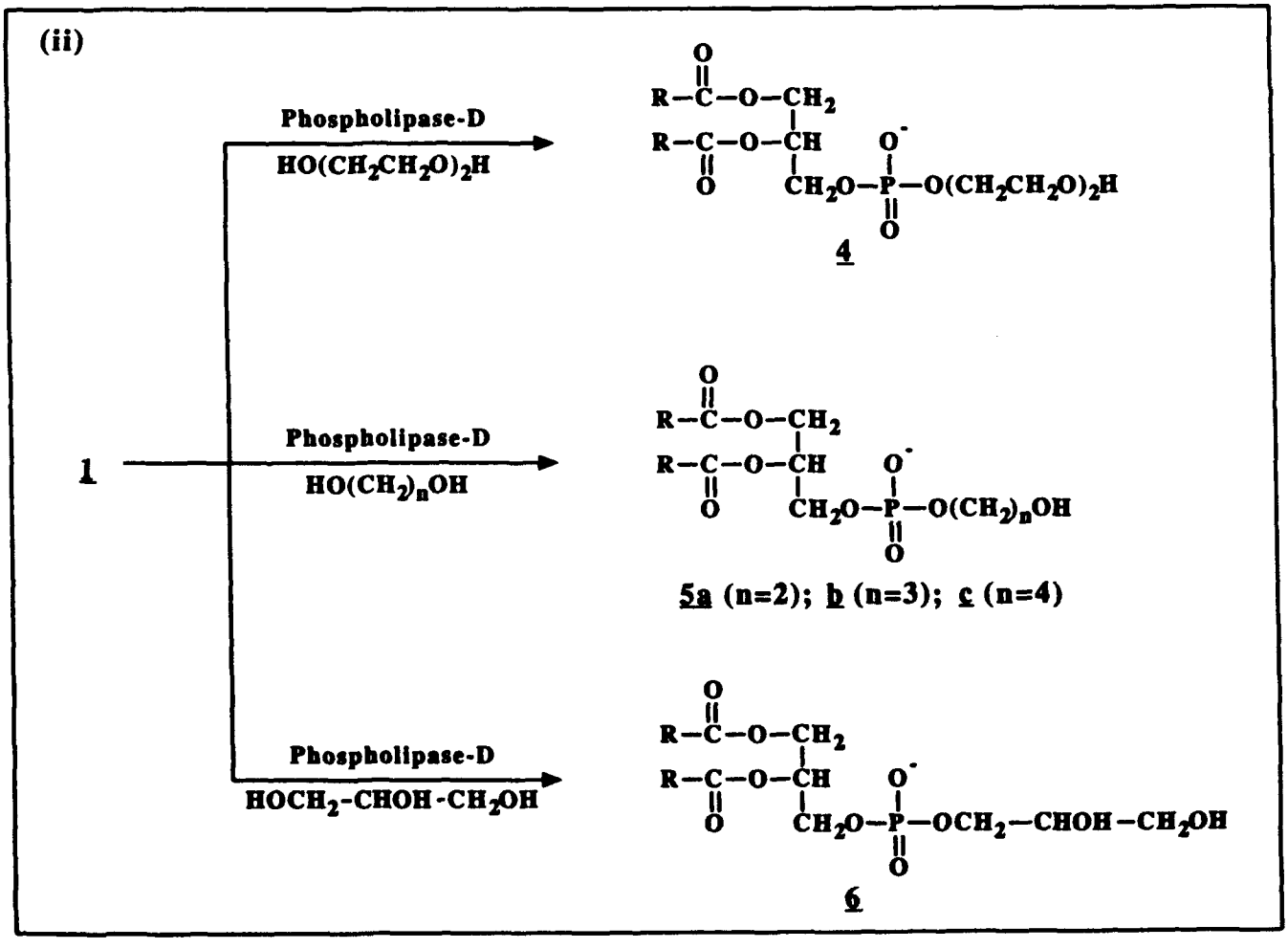
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Phospholipase-D
HO(CH2)nOH
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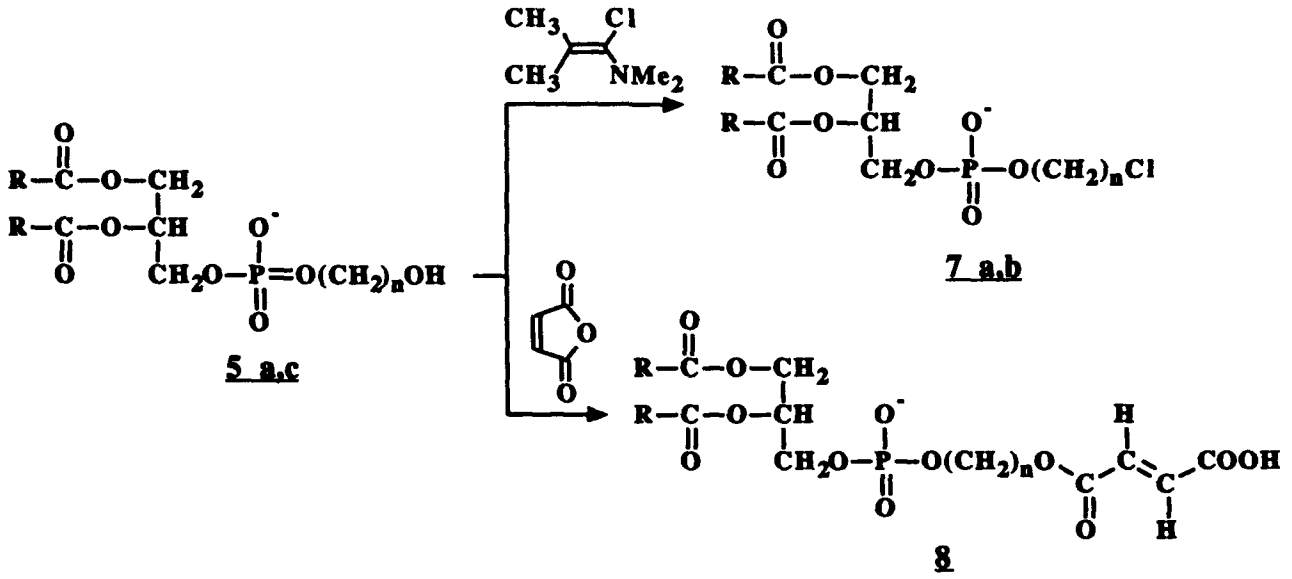
4
5a (n=2); b (n=3); c (n=4)
6

(iii)



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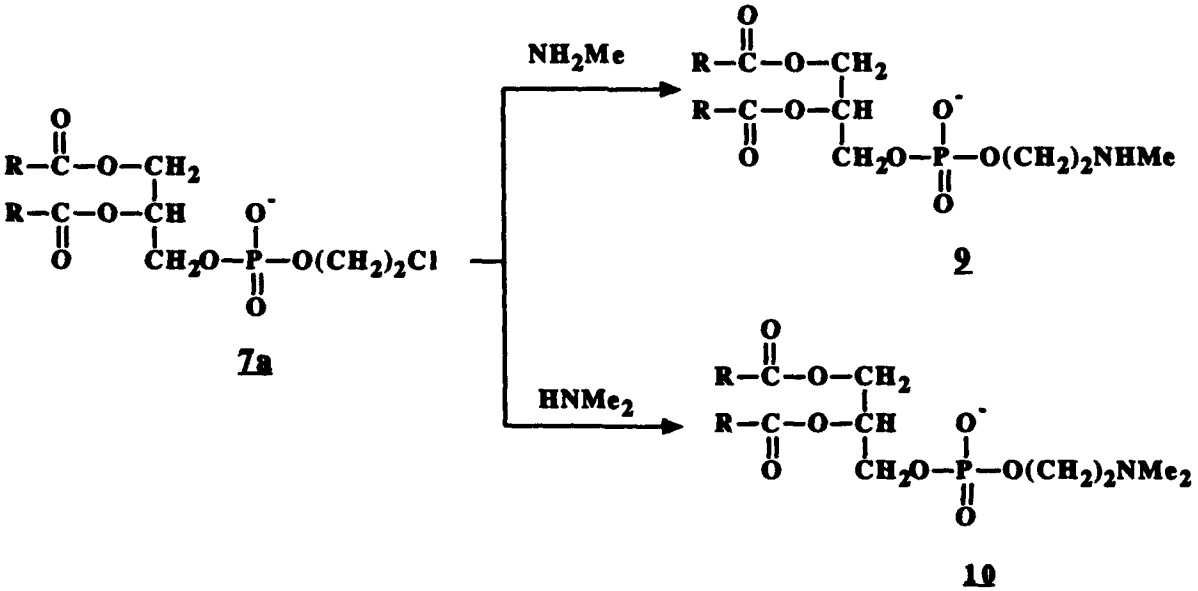

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